#22 B 7/8/03

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Michalopoulos and Bowen	
Serial No.	:	09/455,952	Examiner: Naff, D.
Filed	:	December 7, 1999	Group Art Unit:1651
For	:	A NOVEL LONG-TERM THREE-DIMENSIONAL TISSUE CULTURE SYSTEM	

DECLARATION OF DR. GEORGE K. MICHALOPOULOS AND WILLIAM C. BOWEN UNDER 37 C.F.R. '131

Assistant Commissioner for Patents
Washington, D.C. 20231

We, GEORGE K. MICHALOPOULOS AND WILLIAM C. BOWEN, do declare:

1. We are co-inventors of the invention disclosed in the above-identified application. The invention was concieved and reduced to practice in the United States prior to January 1999. The experimental data described below was generated in the United States.

2. The invention disclosed in the above identified application relates to a novel tissue culture system that provides for the long term culture of proliferating hepatocytes that retain hepatic function. Specifically, the invention relates to methods and compositions for *ex vivo* culturing of hepatocytes and non-parenchymal cells on a matrix coated with a molecule that promotes cell adhesion, proliferation or survival. The hepatic cell culture system can be used to form bio-artificial livers for use in subjects having a hepatic disorder.

3. Prior to January 1999, experiments were conducted to test whether hepatic cells and nonparenchymal cells obtained from liver tissue could be cultured together for hepatic reconstruction. The data presented below demonstrates successful culturing of hepatocytes and nonparenchymal cells that reproduce the hallmark structures of hepatic histological architecture while maintaining differentiation and the capacity to proliferate.

4. Rat hepatocytes were isolated from male Fischer 344 rats by an adaptation of Seglen's calcium two-step collagenase perfusion technique. Typically a 3% contamination with non-parenchymal cells is seen in this isolate. The freshly isolated hepatocytes were added to roller bottles (850 cm^2 surface). Each bottle contained 18.7 x 10^6 beads and 210 x 10^6 freshly isolated hepatocytes in 250 mL of HGM medium supplemented with HGF (20 ng/ml) and EGF (10 ng/ml). The bottles were rotated at a rate of 2.5 rotations per minute and kept in an incubator maintained at 37 °, saturated humidity, and 5% CO_2.

5. Exhibit C depicts thin sections of cells on beads in roller bottle cultures at day 15 after isolation, stained with toluidine blue. (A) The bead is seen as a hollow space in the center of the cell cluster. Gray material around the bead represents dense type-1 collagen deposition. The collagen surrounds and embeds connective-tissue-derived nonparenchymal cells. Cells with hepatocyte morphology surround the connective tissue core. (B) Similar as in A. The epithelial cells with hepatocyte morphology form an eccentric growth over a foundation of connective tissue cells. It is noteworthy that multiple microvilli have formed over the hepatocytes present on the surface.

6. The bead clusters containing cells were isolated from suspensions obtained from the roller bottle cultures. Enrichment for clusters was obtained by allowing for 2 minutes of unit gravity sedimentation. The bead and cell clusters were mixed with Matrigel (Collaborative Research). Bead clusters with cells were allowed to settle whereas beads without cells stayed mostly in suspension. The supernatant was aspirated leaving the clusters in the bottom of the tube. The process was repeated three times. Clusters suspended in medium were mixed with Matrigel at a volume ratio of 1:4 (medium plus beads:Matrigel). Approximately 50 to 100 bead clusters were randomly embedded in Matrigel.

7. Exhibit D depicts cellular and matrix immunohistochemistry of cultures in Matrigel. Staining by immunoperoxidase. A, B, C and D show stains for desmin, collagen types I, III and IV respectively. Desmin-positive stellate cells are interspersed in close proximity to the hepatocytes. Collagen type III shows the strongest immunohistochemical response. Collagen type IV often formed basement membrane structures surrounding hepatocytes arranged in acinar or ductal configurations (arrow).

8. The data presented in Exhibits C and D demonstrates that hepatocytes in long term roller bottle cultures enter into a stable phenotype in terms of morphology and that in the presence of HGF, EGF and Matrigel the cultures are capable of undergoing complex morphogenetic transformations leading to the formation of ducts and plates. The growth in three dimensions forms ducts and sheets of mature hepatocytes surrounded by non-parenchymal cells. Proliferation and cell differentiation is very high in these cultures.

9. We hereby declare further that all statements made herein by our own knowledge are true and that all statements made on information and belief are believed to be true and further that we make these statements with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under ' 1001 of Title 18 of the united States Code and that such willful false statements may jeopardize the validity of the application of any patent issuing therein.

Dated: 6-12-03

G Michalopoulos
Dr. George K. Michalopoulos

Dated: 6-12-03

William C Bowen
MR. ~~DR.~~ William C. Bowen

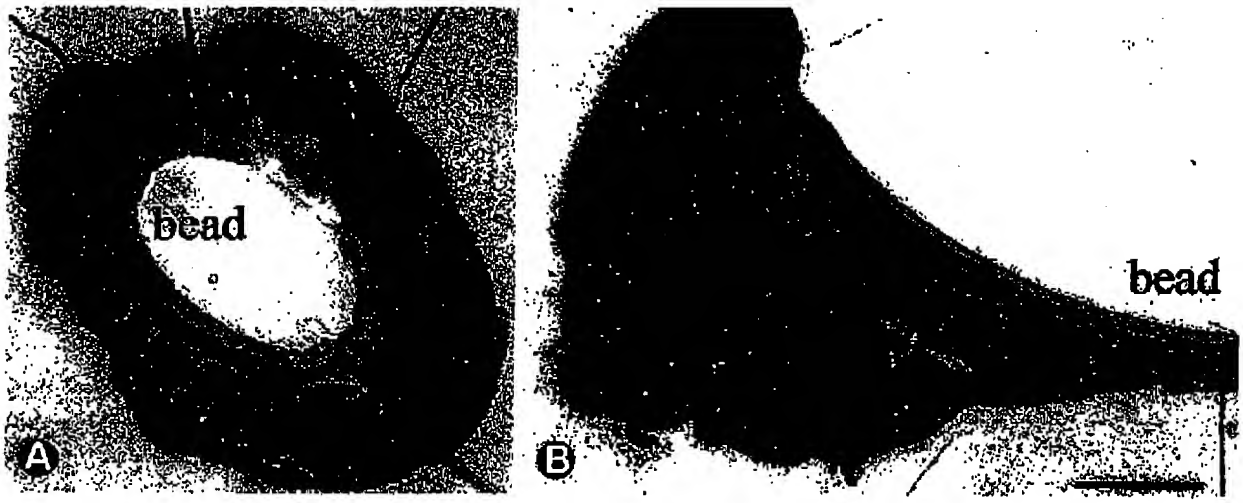

EXHIBIT C

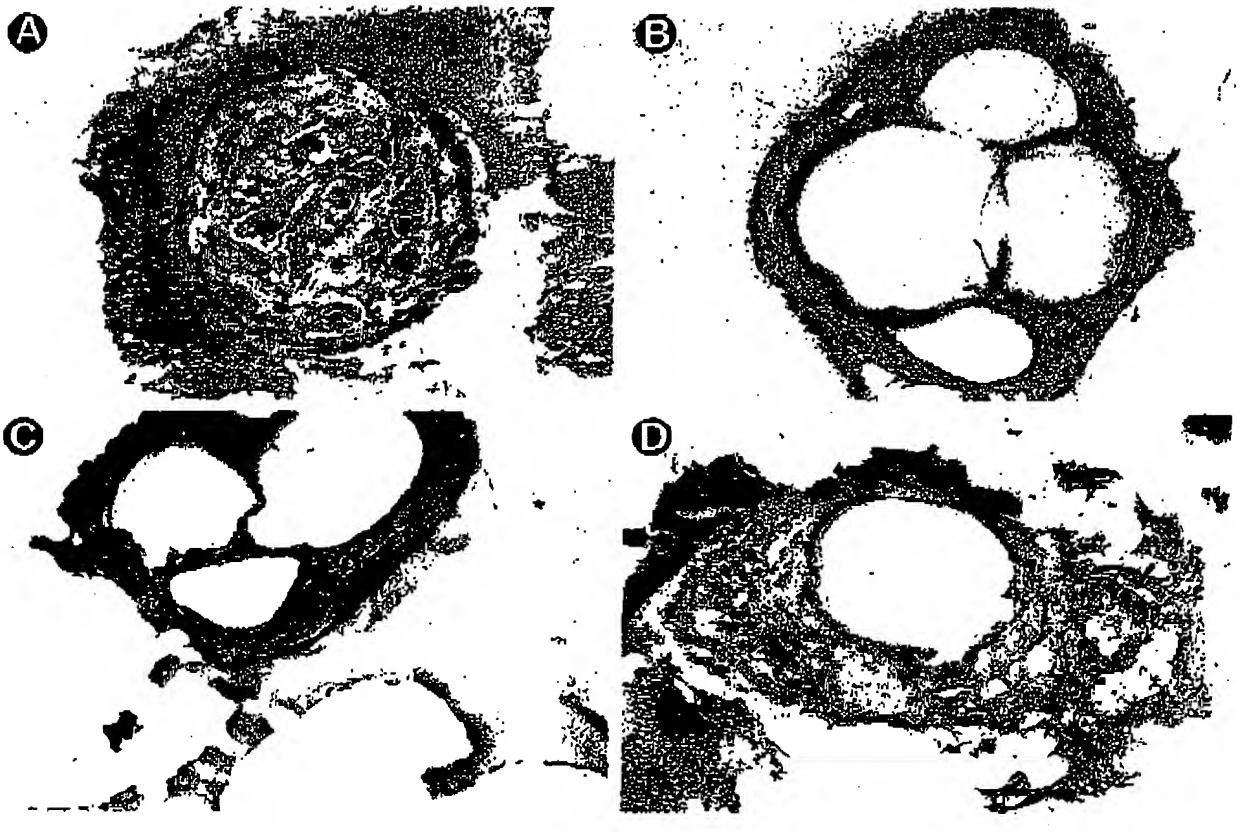

EXHIBIT D